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A Polymorphism in a let-7 microRNA Binding Site of KRAS in Women with Endometriosis

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 29 August 2011

Thank you for the submission of your manuscript "A Polymorphism in a let-7 microRNA Binding Site of KRAS in Women with Endometriosis" to EMBO Molecular Medicine. We have now heard back from the three referees whom we asked to evaluate your manuscript and they find the topic of your manuscript potentially interesting. However, they also raise significant concerns on the study, which should be addressed in a major revision of the manuscript.

Importantly, all reviewers highlight that the description of experimental procedures, rationales, and results must be improved. Of special note in this regard are the patient description, cell proliferation assay, statistical analyses as well as the siRNA experiments.

Given the balance of these evaluations, we feel that we can consider a revision of your manuscript if you can convincingly address the issues that have been raised within the time constraints outlined below.

Revised manuscripts should be submitted within three months of a request for revision. They will otherwise be treated as new submissions, unless arranged differently with the editor.

I look forward to seeing a revised form of your manuscript as soon as possible.

Yours sincerely,

Editor
EMBO Molecular Medicine

***** Reviewer's comments *****

Referee #1:

This manuscript describes the frequency of a polymorphism in the 3' non-coding region of the human KRAS gene which is a binding site for the let-7 microRNA family. This variant polymorphism has a frequency in the general population of approximately 7%. However, and very interestingly, these authors describe a significantly elevated frequency of the variant in patients with well documented endometriosis. This is an extremely significant finding and as the authors correctly claim, gives a mechanistic explanation for aberrant KRAS expression which has been implicated in models of endometriosis. Specifically, 2 papers the authors cite (Dinulescu et al, 2005 and Cheng et al, 2011; citations 16 and 17) have reported that activating mutations in KRAS promote endometriotic lesion formation. However, in human studies of patients with endometriosis no mutations in the RAS gene have been identified. The data presented in this manuscript reconciles this apparent contradiction as the variant RAS allele leads to elevation in KRAS and it is very plausible that this would lead to enhanced endometriotic lesion formation or growth.

I am therefore from the outset well disposed to, in deed enthusiastic about, this paper as it has the potential to make a very substantial contribution to the endometriosis field. It would also be of general interest as it could also serve as a powerful example of how polymorphisms in micro RNA binding sites can lead to functional differences which are implicated in the pathogenesis of significant conditions.

However, while I am enthusiastic in principle about the paper and some of the data contained within it - it has significant, although rectifiable deficiencies and these are listed below.

1. The authors need to take much more care in describing the experiments and the results. Specifically they frequently say they have used endometrium in a particular phase of the study when in fact they have used isolated endometrial stromal cells. An example of this would be in the paragraph describing 'the effect of LCS6 variant allele on KRAS and let-7 expression' in the Results. Here it is claimed endometrium from women with endometriosis with and without the variant allele was used. However, in both the Methods and the figure legend (figure 1) which describes this experiment it is clear that endometrial stromal cells have been used. This must be corrected.

2. A similar problem is evident in the data relating to figure 5. For example in the Methods section describing the 'murine endometriosis model' and the 'immunohistochemistry' section which immediately follows it, the first states that 'endometriosis was created using endometrial cells from subjects ...' whereas the second states 'mouse kidneys containing transplanted hESC that had either ...' Again contradictory descriptions.

3. The cell proliferation data (figure 4) is problematic in a number of ways.

a) Firstly this is described as a 'pulse-chase' this is incorrect, there is no chase.
b) In the Methods section describing this the authors claim to have used 'FixDenat solution' however this is not a component of the kit they claim to have used.
c) The authors need to be clearer as to how many replicates they actually use and from how many different patients. The last line of the Methods section relating to this is unclear.
d) The most significant problem with this proliferation data is apparent from the results ('cell proliferation and invasion'. In this paragraph the authors claim that a 39% reduction of BrdU labelling in the variant containing endometrium (or should that be endometrial stromal cells?) is indicative of increased proliferation of the endometrial stromal cells with the variant allele. This appears to me to be incomprehensible. A reduction of BrdU labelling would indicate a reduction in proliferation of these same cells. This is confusing at the very least and the discrepancy needs to be resolved. Clearly if it turns out that the cells with the variant allele do indeed proliferate more slowly then this would not be supportive of their hypothesis and would weaken the paper, possibly fatally.

4. In figure 3 the authors present data investigating the functional consequences of the KRAS variant as measured by luciferase reporter activity. This is interesting data as it is directly and functionally relevant but has a number of problems as outlined below.

a) The description of the construct used is inadequate. Specifically, the authors need to give sufficient detail to enable the reader to know precisely which portions of the 3' UTR have been used. Incidentally the reference cited to support this (36) is completely inappropriate at this point.
b) The specific sequence of the engineered siRNA used to target the variant allele needs to be given. Without this information the work can not be replicated nor compared to other studies.
c) In the figure legend the author's state that their reporter is 'KRAS variant driven' this is inaccurate since it is the 3' UTR they are investigating not the promoter region.
d) In the figure itself the y axis is labelled 'absorbance'. This is incorrect they are describing relative luciferase activity.
e) The most significant error relating to this particular portion of the data is in the Discussion (paragraph 2) where they state rather extravagantly 'loss of the normal LCS-6 let-7 binding site increases transcription and translation of KRAS'. They have no data to support this assertion. They do have data however to support the claim that alteration of let-7 binding alters luciferase activity in an appropriate reporter cell.

5. The data presented in figure 5 (endometriotic lesions in the nude mouse model) is interesting and pretty convincing. However, it too has a few problems which need to be addressed.

a) The description of which cells and how these were prepared prior to transplant is unclear. Indeed, in the legend again they appear to be claiming they used human endometrial stromal cells, if that is the case then one wonders how the glandular structures formed.
b) The description of their PCNA staining is very poor. In the Results they claim 'more intense nuclear staining' and in the legend 'more prominent'. Both these descriptions are incorrect. What they really mean is more cells stained positive in the lesions derived from cells with the variant allele. They do appear to have counted positive cells as in the legend some percentages are given. However, the description in the Methods and Results relating to this is extremely poor.
c) PCNA is a proliferation marker and has little to do with 'reparation'.
d) The authors should check whether the scale bar does indeed represent 25 micrometres in every panel presented, I suspect it may not.

6. Throughout the manuscript the authors give the relative percentages of whatever variable they are measuring and an associated P value. They rarely (if ever) give standard deviations, standard errors or confidence intervals. This needs to be corrected throughout the manuscript.

7. In the Methods section the authors do describe the statistical tests they have used. However, they appear to have used a t-test but do not indicate that the data sets to which this was applied to was checked to ensure it had a normal distribution. With low n values it is unlikely that they could say this with certainty and they may be more prudently advised to use a non-parametric test (which they did for the immunohistochemical analysis).

8. From the list above you will note there are a large number of rather trivial and annoying errors which would be relatively easy to correct. However, my initial enthusiasm about this paper is considerably diminished by these numerous errors. They do not fill this reviewer with confidence about the care with which the work was actually carried out. Nonetheless all of them, with the possible exception of the problem with the proliferation assay can be resolved relatively easily. Given the potential interest and importance of the fundamental observation in the paper this is somewhat disappointing.

Referee #2:

Summary:
In 2005, Dinulescu et al have shown that genetically engineered mice carrying the mutant K-Ras oncogene resulted in pelvic endometriosis. The submitted manuscript by Grechukhina et al. provides exciting human data that expand upon the earlier studies in rats (Dinulescu) to help discern the role

of a polymorphism in the 3'UTR of KRAS. The data are clearly presented and the discussion is likewise cogent and tremendously exciting for the field of endometriosis that has seen decades of study without a unifying pathophysiologic theory. This study goes a long way towards tying up some loose ends (i.e., why almost uniform retrograde menses doesn't translate into greater endometriosis rates; a link between endometriosis and ovarian cancer). Their fundamental findings are as follows:

(1) K-Ras microRNA variant found in 31% of all cases of endometriosis (n=150) (compared with 5.8% reported incidence for the general population)

(2) Those with K-Ras variant more often presented with infertility while those with the wild-type K-Ras allele usually presented with pain and dysmenorrhea

(3) K-Ras mRNA and protein expression was increased in the eutopic endometrium from women with the K-Ras variant

(4) Human endometrial stromal cells with the K-Ras variant lead to elevated K-Ras protein expression and increased proliferation/invasion

and (5) Using the murine model the K-Ras variant implants revealed increased proliferation and diminished progesterone receptor expression

The plausibility of targeting mutant K-Ras miRNA in the treatment of endometriosis clearly would be a long-term goal of these findings. Zhao et al have written "If KRAS does play a role in the aetiology of endometriosis in women, it may be through somatic mutations in endometriotic lesions." This manuscript does an excellent job at addressing the involvement of K-Ras-associated gene variation in the pathogenesis of endometriosis. My comments below serve as suggestions to improve the strength of this paper.

1. Introduction, page 5, lines 3-10:

It would help to add this reference:

Treloar SA, Wicks J, Nyholt DR, Montgomery GW, Bahlo M, Smith V, Dawson G, Mackay IJ, Weeks DE, Bennett ST, Carey A, Ewen-White KR, Duffy DL, O'connor DT, Barlow. Genomewide linkage study in 1,176 affected sister pair families identifies a significant susceptibility locus for endometriosis on chromosome 10q26. Am J Hum Genet. 2005 Sep;77(3):365-76.

2. Introduction, page 5, lines 22-23:

It would help to add this reference:

Cheng, Ching-wen, et al. Activation of mutated K-ras in donor endometrial epithelium and stroma promotes lesion growth in an intact immunocompetent murine model of endometriosis. J Pathol. 2011 Epub ahead of print.

3. Introduction, page 5, lines 25-30:

It would help to add this reference:

Zhao ZZ, Nyholt DR, Le L, Martin NG, James MR, Treloar SA, Montgomery GW. KRAS variation and risk of endometriosis. Mol Hum Reprod. 2006 Nov;12(11):671-6.

4. Introduction, page 6, lines 13-18:

The authors note the relationship between this SNP and ovarian cancer but since they found an increase in K-Ras protein expression for eutopic endometrium in those with the K-Ras variant what about the RR of endometrial carcinoma in these women? K-Ras proto-oncogene mutations are detected in approximately 10-30% of endometrioid carcinomas. Do the authors know if there is any relationship between women with endometriosis and endometrial cancer?

5. Results, page 7, lines 47-52:

The authors provide the distribution within stages III and IV but could the authors supply the breakdown for stages I and II? [Appendix would be sufficient.]

Also, please specify if possible if the women were heterozygous or homozygous for the variant allele.

6. Appendix, page 29, statistical analysis:

I think a post-hoc test (i.e., Bonferroni) might be necessary for some of their statistics but I defer to a statistician for the definitive assessment.

7. Appendix, page 24, line 56:

For those women without endometriosis used as controls did they have laparoscopic-proven absence of disease? And did they have some other benign gynecologic disorder (i.e., fibroids?). How many were in this control group? This should be delineated.

8. References, page 20, line 35:

The first author was not included in reference no. 16: Daniela M. Dinulescu.

9. Figure 1:

It would be helpful to note in the legend for this figure that these endometrial stromal cells were from eutopic endometrium rather than an endometriotic lesion or endometrioma. Also, wouldn't having columns for the CONTROL GROUP eutopic endometrium, WT v. variant, (as in Figure 2) be elucidating in this figure as well? In fact, I don't believe the manuscript mentions what was the incidence of the K-Ras microRNA variant for their control subjects. This would be valuable information.

Please note the number of women who provided samples in each group for the figures (i.e., Figures 1 and 2).

SNP names from dbSNP should be provided for all variants examined.

Referee #3 (Comments on Novelty/Model System):

The overall quality of the manuscript is poor. Due to insufficient information, data cannot be fully evaluated (please see the comments to authors). For example, the rationale and protocol for the in vitro BrdU pulse-chase experiment are not fully explained. The total amount of BrdU that is incorporated into ESCs should not decrease by proliferation. The lower BrdU level should indicate a lower proliferation rate. The BrdU concentration/nucleus decreases as cells proliferate. Nevertheless, to measure the time-course change in BrdU concentration/cell, the BrdU concentration at time 0 must be determined. Another example is the xenograft experiment. It may have been performed without consideration for the hormone milieu of the host mice.

Referee #3 (Other Remarks):

Association of the KRAS-variant allele that lacks a let-7 binding site on the 3' UTR with certain cancers has been previously reported. In this study, the authors addressed the link between the KRAS variant and pathogenesis of endometriosis. The frequency of the KRAS variant allele among 132 endometriosis patients was higher than the previously reported frequency among the general population, suggesting that the presence of the KRAS variant allele is a risk factor of endometriosis.

In cultured endometrial stromal cells, the variant KRAS allele was associated with an elevated KRAS level, reduced let7 levels and a high proliferation and migration rate. In a mouse xenograft study, the KRAS-variant allele appeared to be associated with high proliferation rate and low progesterone receptor level. Based on these observations, authors concluded that the polymorphism of a let-7 binding site on 3'UTR of KRAS increases the risk of endometriosis through an upregulation of KRAS.

General Comment
The link between the KRAS polymorphism and endometriosis is intriguing. However, validation of the data is difficult because the details and rationale of the experimental procedures are not sufficiently provided.

Specific Comments
1. The association analysis (Table 1) seems to be incomplete.
a . The average number of years from the diagnosis of endometriosis to the time of study should be calculated separately for each genotype.
b. That the ratio of peritoneal endometriosis to total endometriosis (peritoneal + ovarian) is higher among patients carrying the variant allele (44.9%) than patients without the variant allele (27.5%) should be discussed. The symptoms of endometriosis may depend on the site and type of active lesions [Hum Reprod. 2007; 22(1):266-7], thus the difference in the frequency of peritoneal versus ovarian endometriosis may contribute to the significant difference in the frequency of infertility, severe pain, dysmenorrhea and dyspareunia between two genotypes.

2. This study focused exclusively on the stromal component of the endometrium. The rationale for this should be explained. Please cite papers demonstrating that the in vitro proliferation and invasion activities of stromal cells are correlated with the potential of endometrial tissues to form endometriosis lesions.

3. Please provide a detailed description of the cell extraction and culture procedure.
c. The zygosity of the hESCs for variant KRAS allele should be specified. Since only 7 subjects were homozygous for the variant allele, the hESC were presumably extracted from heterozygous patients. Nevertheless, the information is missing.
d. Was the absence of the variant LCS6 allele in the control hESCs confirmed? The information is missing.
e. Please include the number of endometrial biopsies collected from the control subjects (non-endometriotic women), and the frequency of the variant allele among the control subjects.

4. Please provide a detailed description of the proliferation assay protocol, including the number of subjects/hESCs used for each group. Why and how was the in vitro "pulse-chase" assay performed? Current data do not sufficiently provide proof that the proliferation rate of cultured hESCs was higher with the variant KRAS allele.

5. The higher let-7 levels in the hESCs with the variant KRAS allele cannot be explained by the loss of a let-7 binding site on the 3' UTR. This result can be interpreted that the KRAS variant allele increases the risk of endometriosis only in combination with another factor that decreases let-7 levels.

6. Please provide a detailed description of the protocol for the murine endometriosis model. The cited link is for the transplantation of islet cells into kidney capsule, and it doesn't provide sufficient detail of the experimental protocol for endometrial study.
f. Please describe the method that was used to control the hormone levels in host mice. Proliferation rate and gene expression of endometrial cells are regulated by steroid hormones, and the levels of ovarian steroids in female mice change dramatically through the estrus cycle. For example, expression of the progesterone receptor is estrogen dependent, thus it is high at the proestrus and low at the diestrus stages.
g. While cell culture studies were performed only with stromal cells, endometrial implants contained both stromal and glandular cells. The detailed protocol for the preparation of transplants, including the ratio of epithelial and stromal cells, should be provided.
h. The rationale for kidney capsule transplantation should be explained. The phenotypes of the endometrial tissues can change when they are growing on the peritoneum [Hum Reprod Update.

2006;12:641-9]. For this reason, the peritoneal cavity has been used for the transplantation site of human endometrial tissues in induced endometriosis models [Hum Reprod; 1999 Dec;14(12):3107-11, 2001 Aug;16(8):1736-43, 2004 Jun;19(6):1265-71, 2005 Feb;20(2):350-8]. Contrarily, the kidney capsule has been used to study the function of normal endometrium, not endometriosis [Proc Natl Acad Sci USA. 2007 Feb 6;104(6):1925-30, Hum Reprod. 2009;24(8):1960-7].

7. The method and actual data of quantitative immunohistochemical analysis is missing. The p-value itself cannot be the data.

1st Revision - Authors' Response 11 October 2011

We would like to thank the reviewers for the careful critique and valuable comments. We appreciate the thoughtful reviews and have addressed all of their concerns. This process has resulted in a significantly improved manuscript.
The revised manuscript includes additional experiments including a second measure of BrdU incorporation and the inclusion of more controls.
We agree with all of the comments of the reviewers and have made all of the changes suggested by each of the reviewers. Specifically we have addressed the reviewer comments as follows:

Referee #1:
We thank reviewer 1 for the very positive comments and stating that the paper ìhas the potential to make a very substantial contribution.î We agree that the manuscript had ìsignificant, although rectifiable deficienciesî and have corrected them all as suggested.
1. The reviewer points out that we used the term endometrium when in fact we used stromal cells. In these experiments we used cultured human endometrial stromal cells isolated from eutopic endometrium of women without endometriosis or with endometriosis carrying WT or variant KRAS allele. We have clarified the cells used throughout the manuscript.

2. Similarly, we have substituted the term endometrial cells with the more appropriate term cultured hESC to avoid misunderstanding.

3. Cell proliferation assay:
We thank the reviewer for advice on this matter. The original BrdU labeling experiments were conducted differently from the method suggested by the manufacturer in the kit protocol ñ we did track the dilution of BrdU label after a pulse treatment of the cells with the labeling solution followed by incubation in the serum containing medium for 48h. However, following your comment, we repeated proliferation assay according to the kit protocol which we now thoroughly describe in the edited manuscript. The results were consistent with our previous data, showing a significantly higher proliferation rate (71% increase) in the cultured hESC from women with endometriosis heterozygous for variant KRAS allele compared to hESC homozygous for the WT KRAS allele The assay was performed 3 times in triplicate using samples obtained from five separate subjects.

4. We have edited the method section concerning the luciferase reporter construct.
a. We have added the precise information on the structure of the reporter and corrected the erroneous reference.
b. We also added the specific sequence of the engineered siRNA that was used to target the variant LCS6 in 3 UTR of KRAS.
c. We have removed the phrase ìKRAS driven luciferase activityî from the figure legend as well as from the text.
d. We have corrected the axis label in figure 4. The label is now called ìRelative luciferase activityî.
e. We have removed from the discussion a controversial statement regarding the increase of transcription and translation of KRAS as a direct result of loss of normal LCS6 let7 binding site. We agree that our data is only suggestive; instead we say that increased luciferase activity was seen after loss of let-7 binding.

5. Murine model:
a. We have added a more detailed description of the way we prepared the cultured hESC as well as the procedure itself. In the murine endometriosis model we used cultured human endometrial cells. Prior to the cells being plated the mixture of endometrial stromal and glandular cells was passed through 40 um sieve. Dispersed stromal cells pass through the sieve while non-dispersed epithelial cell remain on the upper surface of the sieve. Stromal cells were then cultured in serum containing medium and passaged 3-5 times before they were used for transplantation experiment. A large number of studies have been recently carried out in order to understand the mechanisms of endometrial restoration in the estrus/menstrual cycle. Most investigators agree that a stem cell is involved in this process; recently Cervello et al have demonstrated the formation of human endometrium (glands and stroma) from an endometrial stromal progenitor stem cell in the kidney capsule of immunodeficient mice (Cervello I et. al.2011 PLoS One). The fact that our cells did form tissue composed of both epithelial and stromal components lends support to the hypothesis that stromal and glandular cells may originate from progenitor cells in human endometrium. Alternatively, small numbers of epithelial cells are known to pass through the sieve and some may have persisted in culture.
b. We have edited the results section concerning the description of proliferation marker PCNA and added the % of positively stained nuclei for all the markers.
c. We have removed the word ìreparationî from the description of this proliferation marker.
d. We thank the reviewer for noting the inaccuracy of our scale bar and have corrected the error.

6. We have added SEM (Standard Error of the Mean) values throughout the manuscript.

7. Following your suggestion we have use non-parametric Mann-Whitney test to calculate p values for Dual Luciferase Reporter, proliferation and invasion assays.

Referee #2:

We thank the reviewer for stating that ìthe data are clearly presented and the discussion is likewise cogent and tremendously exciting for the field of endometriosis.î
1. We have added the following reference:
Treloar SA, Wicks J, Nyholt DR, Montgomery GW, Bahlo M, Smith V, Dawson G, Mackay IJ, Weeks DE, Bennett ST, Carey A, Ewen-White KR, Duffy DL, O'connor DT, Barlow. Genomewide linkage study in 1,176 affected sister pair families identifies a significant susceptibility locus for endometriosis on chromosome 10q26. Am J Hum Genet. 2005 Sep;77(3):365-76.

2. We have added the following reference:
Cheng, Ching-wen, et al. Activation of mutated K-ras in donor endometrial epithelium and stroma promotes lesion growth in an intact immunocompetent murine model of endometriosis. J Pathol. 2011 Epub ahead of print.
3. We have added the following reference:
Zhao ZZ, Nyholt DR, Le L, Martin NG, James MR, Treloar SA, Montgomery GW. KRAS variation and risk of endometriosis. Mol Hum Reprod. 2006 Nov;12(11):671-6.

4. We are unaware of any studies linking endometriosis to endometrial cancer. We speculate that activating K-Ras mutation leads to distinct disease from the let-7 binding site mutation.

5. The majority of the women tested positive for the variant KRAS allele were heterozygous for it with only 7 subjects being homozygous. This equates to 5.3% of all subjects tested and 17% of KRAS-variant positive cases.

Women with endometriosis stage III and IV constituted the majority of the group. The smaller numbers of stage I and II do not allow us to make any statistical conclusions about the frequency of different symptoms in this group. However it is an interesting question and we will be looking at this as we expand our numbers.

6. We have consulted with a statistician. As recommended by reviewer one, we used non-parametric tests where appropriate and also corrected for multiple comparisons.

7. Endometrium from 6 women without surgical evidence of endometriosis but with possible other benign gynecological conditions (e.g. fibroids, benign ovarian cysts) who tested negative for the presence of variant allele was used as controls.

8. We have added author Daniela M. Dinulescu to reference 16 where her name was accidentally deleted.

9. Following your advice we edited figure 1, adding a control group column. The KRAS mRNA expression is significantly lower in endometrial stromal cells extracted from eutopic endometrium of women without endometriosis which can be attributed to the higher levels of let-7 in these cases.

10. The accession number for this polymorphism is rs61764370, which was included in the text of the manuscript.

Referee #3

We appreciate that reviewer one found the data intriguing and have clarified details of the rational and experimental procedures as requested.

1. We thank the reviewer for the interesting observations and suggestions. We have included the requested information and analysis in the revised manuscript.

a. The number of years from diagnosis to inclusion in the study is now included for each genotype in table 1.

b. The reviewer makes a thoughtful and important observation. The variant allele is associated with a higher incidence of peritoneal endometriosis. This is discussed in paragraph 1 of the revised discussion.

2. It has been previously shown by other investigators that the stromal component of endometrium plays a crucial role in regulating endometrial growth and controlling epithelial growth. Moreover, it is well established that a defect in the stromal cells is responsible for defective estradiol metabolism in eutopic and ectopic endometrial tissue in patients with endometriosis. We have cited papers demonstrating differential stromal cell proliferation and invasion in endometriosis.

3. We have edited the methods section describing the cell extraction and culture procedures in more detail.

c. For all experiments hESC heterozygous for variant allele were compared to normal hESC or hESC from women with endometriosis but homozygous for the WT KRAS allele.

d. Endometrium from women without surgical evidence of endometriosis and who tested negative for the presence of variant allele was used as controls.

e. Among 10 subjects used as controls, none tested positive for KRAS variant allele.

4. Taking into account your concern and that of reviewer 1 regarding the proliferation assay, we repeated the experiment with the protocol recommended by the manufacturer. The data confirm our original results. The proliferation rate of cultured hESC from women with endometriosis heterozygous for variant KRAS allele was significantly increased compared to hESC homozygous for the WT KRAS allele. We thoroughly describe the protocol, including number of subjects and replicates, in the revised version of the manuscript.

5. We agree with the reviewer that we have not provided a mechanism to explain how the variant allele could explain the altered let-7 levels. We have edited the text to state that hESC from women with endometriosis exhibited lower levels of let-7 family miRNA expression that co-existed

with the presence of the KRAS variant allele and could be a result of yet undiscovered feedback mechanism or due to another independent factor. However, low let-7 in the presence of tumors with the KRAS-variant has also been documented, suggesting that there is most likely a feedback loop that leads to this relationship.

6. Following your recommendation we described the murine endometriosis model in more detail.
f. Prior to surgeries, all mice were synchronized by vaginal cytology. All experiments on mice were performed in the proestrous phase.
g. Reviewer 1 also appropriately expressed a similar concern. Please see the response 5a to reviewer. Others have shown the regeneration of a complete human endometrium using the mouse kidney capsule model from a progenitor stem cell population. While we believe the formation of endometrial epithelium and stroma indicates the presence of these cells, we do not exclude the possibility of contaminating epithelial cells in our transplants. The origin of these cells does not alter our conclusions on the role of the KRAS variant.
h. The aim of this experiment was to determine if hESC from women with endometriosis and either the WT KRAS or variant KRAS had different biological properties in vivo. We agree that the phenotype of the endometrial tissue can differ depending on the location of the implant; however most endometriosis originates from retrograde menstruation and ectopic implantation of the eutopic endometrium. We expect the both eutopic and ectopic endometrium to demonstrate differences if the variant allele is present. These differences may be important for the establishment of disease in addition to its behavior once in the ectopic location. We have had experience with both the peritoneal model and the kidney capsule model. In this set of experiments the practicalities favored the use of the kidney capsule. Here the human endometrial samples had first to be assessed for the presence or absence of variant allele, and the mice cycled for transplantation in the proestrus phase, leading us to use cultured cells rather than fresh tissue and the kidney capsule to contain the cells. Further we were able to more accurately assess that equivalent numbers of cells were used and localize the tissue after growth in vivo. Future studies will assess the behavior of the variant endometriois in the peritoneal cavity and response to treatment.

7. We have added appropriate quantitative data (including the % of stained nuclei and p values) of IHC analysis to the results section. The methods section has also been modified.

We thank the editor and reviewers for their insight and helpful comments. We hope that you will find the revised manuscript significantly improved and acceptable for publication in the EMBO Molecular Medicine. Please do not hesitate to contact us if further alterations are required.

2nd Editorial Decision 02 November 2011

Thank you for the submission of your revised manuscript "A Polymorphism in a let-7 microRNA Binding Site of KRAS in Women with Endometriosis" to EMBO Molecular Medicine. We have now received the enclosed reports from the referees whom we asked to re-assess it.

As you will see, the Reviewers acknowledge that the manuscript was significantly improved during revision. However, they still raise concerns. Since we do acknowledge the potential interest of your findings, we would be willing to consider a revised manuscript with the understanding that the referee concerns must be convincingly and conclusively addressed.

Specifically, Reviewer #3 highlights that the endometrial stromal cell cultures should be investigated for stromal and epithelial cell markers to provide information about the quality of the cultures. In addition, he/she highlights that the transplant experiments should be repeated using a method that controls hormone levels. We agree that it would be ideal to perform the suggested experiment, however, we realize that the addition of more data would be time-consuming. Should you be able to provide the data, we would encourage you to include them into the present study. Otherwise, we would strongly encourage you to include a brief discussion of this issue into the manuscript.

Of note, Reviewer #2 highlights that patient numbers should be more prominently mentioned. This could for example be done in the figure legend. In addition, Reviewer #1 points to remaining concerns regarding unclear numbers and statistical analysis. Please see below for an excerpt from our Guidelines on Statistical analysis.

On a more editorial note, immunoblots should be surrounded by a black line to indicate the borders of the blot, if the background is faint. This should be done for Figure 1B.

I look forward to seeing a revised form of your manuscript as soon as possible.

Statistical analysis:
The description of all reported data that includes statistical testing must state the name of the statistical test used to generate error bars and P values, the number (n) of independent experiments underlying each data point (not replicate measures of one sample), and the actual P value for each test (not merely 'significant' or 'P < 0.05').

Yours sincerely,

Editor
EMBO Molecular Medicine

***** Reviewer's comments *****

Referee #1:

This manuscript has been revised in light of my previous comments and I am pleased to see that the majority of these have been addressed satisfactorily. There are however still a few points which should be addressed.

1. In my previous comments (6) I suggested that merely giving the percentage change in the variable of interest and the associated p value was inadequate. This has been partially addressed but there are clear instances where it has not. For example in the Results and figure legend describing the data presented in figure 4 again simple percentage increase and p value are given. From the description of the method used for this it is unclear how many samples have been assayed. The methods state 'the assay was performed three times in triplicate using hESC obtained from at least five different subjects in each group'. There would seem to be little point in repeating the measurements three times if they are performed in triplicate and it is important to know how many subjects were actually used in each group. For the statistical analysis one assumes a t-test has been used but if cells from only five subjects have been used it is very unlikely that the data will be distributed normally. Therefore a non-parametric test needs to be used. The proliferation data described in figure 4 is critical for the flow of the argument the authors are presenting and therefore this data needs to be robust. While the Method and Results are now much clearer than there were in the previous version there is still considerable room for improvement.

2. Similarly in the data presented in figure 5 there is a lack of clarity as to what was counted and how the statistical tests were carried out. The data here are again from a small number of subjects (in this case three mice in each group). The statistical analysis of data obtained from these needs to be done with considerable caution. It is not valid for example to treat separate fields of view on an individual slide as independent samples, the only samples that are truly independent must come individual animals. Therefore counting the positive cells in multiple fields from several microscope slides increases the precision of the measurement from that individual animal but such data should not be used erroneously to increase the apparent 'n' used, it is still three in each group. Given the small P values presented relating to this data I suspect that individual sections or possibly even individual fields of view have been treated as independent samples. This should be corrected or at the very least clarified.

3. A relatively minor and possibly pedantic point which would improve the manuscript would be to

remove the 'expression' from most if not every sentence in which it occurs. This word is frequently redundant and sometimes can imply more than the authors really intend (i.e. direct effects on transcription or translation).

This is much improved than the previous version and I am still enthusiastic about the data contained in it. However, the quality of the description of the experiments and their results still leaves something to be desired. There really should be absolute clarity as to what was done, what was measured and what statistical tests have been used to interpret these data. When reading this paper all of this is just a little bit fuzzy but nonetheless the results are genuinely exciting.

Referee #2 (Comments on Novelty/Model System):

No further comment. My only MINOR residual point is with their reply to item #9 in my review particularly: "Please not the number of women who provided samples in each group for the figures (i.e., Figures 1 and 2)." I don't believe they have done this or addressed this point.

Referee #2 (Other Remarks):

I believe the authors have adequately addressed the various concerns from the three reviewers in this revised manuscript. They have made significant changes that reflect the constructive criticism from said reviews.

Referee #3 (Comments on Novelty/Model System):

The following two observations regarding the transplant experiment require further validation.
1. Endometrial tissues with epithelial glands regenerated from pure endometrial stromal cells.

This issue should be addressed by testing fro the presence or absence of epithelial cells in the ESC culture. If a high number of epithelial cells was present in the culture, the validity of data obtained from experiments utilizing that ESC culture would be in question.

2. Endometrial tissues from women carrying the variant KRAS-LCS6 allele were hyperplastic and contained very low levels of progesterone receptor.

This point should be addressed by repeating the experiment including hormone treatment. The hormone levels in the host mice were monitored by vaginal smear cytology. However, the systemic hormone levels in female mice fluctuate during the day. In addition, variations in the estrogen and progesterone levels between host mice can occur. Hence, the difference in proliferation and PR levels between two genotypes can be due to difference in the hormone levels between host mice.

Referee #3 (Other Remarks):

The authors have properly addressed some of the critical issues raised by this reviewer.
The revised manuscript describes sufficient details of the experimental procedures.
The current manuscript reasonably establishes the association of the KRAS-LCS6 variant allele lacking a let-7 binding site with an increased risk of endometriosis.
However, authors report extraordinary observations that require further investigations. The following claims need to be removed from the manuscript or confirmed by performing the two experiments suggested below.

1. Endometrial tissues with epithelial glands regenerated from pure endometrial stromal cells after 4 weeks of in vivo growth.
Formation of endometrial glands from purified stromal cells is a novel and exciting finding. However, the expression of stromal and epithelial cell markers in the isolated hESCs was not examined. Another possible explanation for the presence of endometrial glands in the endometrial transplants is a contamination of epithelial cells. Based on the histology of transplants presented in

the manuscript, the epithelial fraction was not minor. It raises a question about the quality of hESC cultures used throughout this study. It may have contained endothelial cells as well. The presence of other cell types may affect the average growth and mobility rates of the culture.

Hence, the authors should determine the concentration of CD10 and keratin positive cells in the hESC cultures.

2. Endometrial tissues from women carrying the variant KRAS-LCS6 allele were hyperplastic and contained very low levels of progesterone receptor.
This is also a striking finding with significant clinical implications. The important point is that the kidney transplants were made with cells extracted from normal endometrium, not endometriosis lesions. It means that heterozygosity in a single polymorphic allele alters fundamental functions of the uterus. A significantly reduced level of progesterone receptor in the endometrial cells should increase the risk of infertility and endometrial cancer. Although case control studies have not been able to detect an association between endometriosis and endometrial cancers [Mehasseb ('11) Fertility and Sterility 95, 2228-35], the association between infertility and endometriosis is well established. Hence, this observation may be extremely important.
However, there still is a possibility that this observation was an artifact from the experimental design. According to the materials and methods, the control and KRAS-LCS6 variant endometrial tissues were grown in different mice, and "all surgeries and tissue collection were synchronized, and all mice were operated on in their proestrus phase". It should be noted that the systemic hormone levels are not always constant, even though the vaginal smear indicated proestrus. Hence, the difference in proliferation and PR levels between two genotypes may be due to a variation in the estrogen and progesterone levels between host mice.

For this reason, it would be advantageous to repeat transplant experiment using a better-controlled method.

Additional comments
The experimental results and proposed model are not in complete accord with previous reports. Hence, it is critical for the authors to establish claims by performing the experiments suggested above. The following provides an example of observations that appear to disagree with this study.
1. Although human studies in the past did not examine the KRAS polymorphism, women with the KRAS variant alleles were likely to have been included in these studies considering the high incidence of the variant allele among women with endometriosis (31%). However, most studies did not find a high proliferation rate and low PR levels in the eutopic endometrium of women with endometriosis compared to that of healthy women [Jones ('95) Human Reproduction 10, 3272-9; Jurgensen ('96) Fertility and Sterility 66, 369-75; Nisolle ('97) Fertility and Sterility 68, 912-9; Leyendecker ('02) Human Reproduction 17, 2725-36; Kao ('03) Endocrinology 144, 2870-81]. An increased KRAS level was also not detected in the eutopic endometrium of endometriotic women [Kao ('03) Endocrinology 144, 2870-81].

2. The induced endometriosis model in baboon demonstrated that development of endometriosis lesions alters gene expression in the eutopic endometrium by affecting the immune system [Fazleabas ('06) Methods in Molecular Medicine 121, 95-9]. This suggests that differences in the eutopic endometria of healthy and endometriotic women [Kao ('03) Endocrinology 144, 2870-81] are not the cause, but the effect of endometriosis.

3. While many studies were unable to detect significant difference in the proliferation rate and PR level between the eutopic endometria of healthy and endometriotic women, differences between eutopic and ectopic endometria have been consistently reported [Jones ('95) Human Reproduction 10, 3272-9; Jurgensen ('96) Fertility and Sterility 66, 369-75; Nisolle ('97) Fertility and Sterility 68, 912-9; Leyendecker ('02) Human Reproduction 17, 2725-36; Kao ('03) Endocrinology 144, 2870-81]. It suggests that the unique phenotype of ectopic endometrium develops following the establishment of endometriosis lesions.

3. A previous transgenic mouse model demonstrated a link between the activating mutation of KRAS and endometriosis [Dinulescu ('05) Nature Medicine 11, 63-70]. Hence, it seems to be reasonable to postulate that the loss of let-7 binding to the 3'UTR increases the KRAS expression,

and the high level of KRAS promotes the formation of endometriosis lesions by increasing the rate of proliferation, invasion and survival of retrograded endometrial cells. However, the effect of overexpression of wild-type RAS does not provide an equivalent result to the activating mutation of RAS [Zhang ('01) Nature Genetics 29, 25-33; Singh ('05) The FASEB journal 19, 161-9]. In addition, Kras activity does not appear to regulate PR levels. At least in mouse, an activating mutation in Kras was not shown to affect expression of PR in the uterus [Kim ('10) Journal of Oncology, 2010, 139087].

2nd Revision - Authors' Response 07 December 2011

We appreciate your interest in our work and we believe that we have now thoroughly addressed all the concerns of the reviewers. We thank you and the reviewers for the rigorous reviews and constructive criticism.
Specifically we have revised the manuscript to include a further discussion of the limitations of the mouse model and the cell culture techniques as requested by reviewer 3. In the revised manuscript we have improved and expanded our description of statistical methods for all the experiments as requested by reviewers 1 and 2.
We also have put a black line around the image on figure 1B.
We thank the reviewers' for their positive remarks and valuable comments which we addressed as follows:

Referee #1:
We thank reviewer one for stating that ìthe results are genuinely exciting.î
1. We have ensured that the description of each experiment contains a clear explanation of the number of samples involved, mean +/- standard error of the mean, p value and statistical method used to analyze the data. Specifically we added the absorbance values and exact number of samples used to the description of invasion assay (Fig. 4B). For the statistical analysis of the invasion assay results, as well as the results of proliferation, dual luciferase reporter assays and IHC, the Mann-Whitney U test has been used.
2. For analysis of the IHC results in figure 5 multiple slides from the same animal were averaged to obtain the mean for each biologic replicate. The means obtained from each animal were used to calculate the means presented in the manuscript. However, due to an error in use of the statistics program, each slide was used as an independent sample to assess statistical significance; we have presented the corrected p value in the revised manuscript.
3. We appreciate your comment regarding the redundancy of the term ìexpressionî. We have removed it when appropriate.

Referee #2
We added the numbers of samples used in figs. 1 and 2 to the revised manuscript.

Referee #3
1. The fact that endometrial stromal cells contributed to the formation of both epithelium and stroma is remarkable but not unexpected. According to our previous experience primary endometrial stromal cell cultures do contain a few epithelial cells at passage #1 which are not detectable by passage #3. However we do not exclude the chance of minor epithelial contamination. We have added a description of the cell types present in our culture system to the revised manuscript.
Another explanation for this phenomenon is the presence of endometrial progenitor stem cell among the population of human endometrial stromal cells. These cells can give rise to both stromal and epithelial cell types. This was recently shown by Cervello et. al. (2011 PLoS One) who demonstrated that human endometrium could be regenerated from human endometrial side population cell lines obtained from the epithelial as well as from the stromal compartments. We have added a discussion of this phenomenon to the revised manuscript.
Regardless of the origin of the epithelial cells, all cells in a single lesion (both epithelial and stromal) contain either WT or variant KRAS allele; the differences in the behavior of the lesions reflect the differences in the molecular structure of the 3'UTR of KRAS gene.

2. The reviewer is concerned by the possibility that differences in the hormonal levels between the host animals had an effect on the proliferative characteristics of the lesions as well as their hormone receptor profile. We agree that ovariectomizing the animals with subsequent fixed hormone administration would be more a precise method. However we believe that synchronizing the mice with vaginal smear is an adequate method which has been widely used. We have had extensive experience with this model and in all prior experiments have seen consistent levels of PR in the proestrus phase of animals timed in this way. Moreover the use of intact animals allowed us to see how these cells behave in the environment which more closely mimics normal hormone exposure in women.

We have added a sentence describing the limitations of the model system to the revised manuscript.

Reply to additional comments of reviewer 3:

1. We agree with the reviewer that the existence of differences in proliferation rate and PR expression of eutopic endometrium of normal women and women with endometriosis has been controversial; however several reports are in agreement with our findings. Specifically, Richard O. Burney et al. (Endocrinology 2006;148(8):3814ñ3826) demonstrate enhanced cellular survival and persistent expression of panel of genes involved in DNA synthesis and cellular mitosis in eutopic endometrium of patients with endometriosis compared to endometrium of healthy women. Similar results were obtained by Lusine Aghajanova (Endocrinology, 2010; 151(3): 1341-55). Moreover they also noted a striking dysregulation of progesterone-targeted genes in eutopic endometrium of endometriosis patients. Further the same authors report decreased PR in cells obtained from eutopic endometrium of women with endometriosis compared to controls (Biol Reprod 2011, 84: 801-805). The reviewer also mentions that an increased KRAS was not detected in the eutopic endometrium of women with endometriosis in the study by Kao et al. (Endocrinology, 2003, 144: 2870-81). In that study all the samples were obtained in the implantation window and analyzed with oligonucleotide microarrays. In the implantation window, a time of high progesterone secretion, PR is suppressed in women with or without endometriosis. To avoid PR suppression we intentionally assayed in the proliferative phase. The timeframe of sample collection and limit of a 2-fold increase of gene expression in the Kao study suggest a high chance of missing KRAS overexpression.
2. We agree that endometriosis does affect eutopic endometrium and have published evidence supporting this concept. However eutopic endometrium in women with endometriosis likely also has preexisting intrinsic abnormalities predisposing to endometriosis formation in the first place.
3. We agree that the fully abnormal phenotype of endometriosis is formed after the lesions are established; however there must be inherent abnormalities that lead some women to have an increased risk of generating lesions in the first place. Our data support the concept of preexisting genetic abnormalities in these women, as do the recent GWAS studies referenced in the manuscript.
4. The reviewer questions differences between the KRAS variant described here and the model using either wild type or the activating mutation in Kras. We expect that an abnormally regulated KRAS may have a different phenotype compared to that resulting from forced high levels of expression. Further, we suspect that the compensatory altered regulation of multiple let family members as described here account for some of these differences; however a complete explanation of these differences is speculative at this point.

Thank you for allowing us the opportunity to further revise this manuscript.

We have addressed all of the reviewers' comments and believe that this has resulted in a substantially improved manuscript. We are hopeful that our manuscript is now suitable for publication in EMBO Molecular Medicine. We look forward to hearing from you.

